Effects of dietary phytol and phytanic acid in animals

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ABSTRACT Feeding of phytol in large doses (2–5% by weight in the diet) led to accumulation of phytanic acid in the mouse, rat, rabbit, and chinchilla, the degree of accumulation depending upon the level of dietary intake. The relative concentration of phytanic acid, expressed as a percentage of the total fatty acids, was as high as 20–60% in liver and 30–40% in serum. Phytenic acid, which may be an intermediate in the conversion of phytol to phytanic acid, also accumulated.

When phytol was withdrawn from the diet, tissue and serum concentrations of phytanic acid fell rapidly, which indicates the ability of the normal animal to metabolize phytanic acid readily.

At high dosages in the diet, phytol inhibited growth and caused death within 1–4 weeks. In the mouse, dietary phytanic acid and dietary phytol fed in equivalent amounts were of comparable toxicity. Accumulation of tissue phytanic acid occurred more rapidly when phytanic acid was fed than when phytol was fed in equal amounts.

In none of the animals fed either phytol or phytanic acid were there any signs of neurological defects. Histologic examination of rats fed phytol showed some fat accumulation, glycogen depletion, and karyokinesis in the liver. There were no pathologic changes in the retina or in the peripheral and central nervous system such as those described in Refsum's disease.

KEY WORDS phytol · phytanic acid · phytenic acid · accumulation · rat · mouse · rabbit · chinchilla · branched-chain fatty acids · lipidoses · Refsum's disease

PATIENTS WITH heredopathia atactica polyneuritiformis (Refsum's disease), a hereditary recessive disease affecting primarily nervous system function (3), accumulate phytanic acid (3,7,11,15-tetramethyl hexadecan-1-oic acid) in their serum and tissues (4). This branched-chain fatty acid is ordinarily not detected in the fatty acids of normal serum and tissues, although it has been shown to be present as a very minor component (0.4–2 μg/ml) in human serum (5, 6), in a number of ruminant tissues (7–10), and in butter fat (11–13). By contrast, in patients with Refsum's disease, it can account for as much as 20% of the total fatty acids in the serum and over 50% of the fatty acids in the liver (4, 14–26). Preliminary clinical studies suggest that patients with this disease have a reduced capacity for oxidizing phytol (3,7,11,15-tetramethyl hexadec-2-en-1-ol) and (or) phytanic acid (17, 18). Experiments designed to demonstrate endogenous biosynthesis of phytanic acid in Refsum's disease have been negative (17, 18). On the other hand, orally administered radioactive phytol was readily incorporated into serum phytanic acid in normal subjects and in subjects with Refsum's disease (17–19).

We have previously reported on the conversion of dietary phytol to phytanic acid with accumulation of the latter in the rat (1, 20), findings confirmed by Klenk and Kremer (6, 21). In the present studies, four mammalian species, including conventional and germ-free rats, were fed various doses of phytol for several weeks, and phytol, phytanic acid, and phytenic acid (3,7,11,15-tetramethyl hexadec-2-en-1-oic acid) were determined in serum and tissues. During the course of the studies, the animals were carefully observed for signs of nerve dysfunction and the tissues of some animals were examined for possible structural changes in the peripheral and central nervous system. Further studies on the metabolism of isotopically

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Abbreviations: GLC, gas–liquid chromatography; TLC, thin-layer chromatography.

labeled phytol and phytanic acid are presented in the accompanying paper (22).

METHODS

Preparation of Diets

Animals were fed commercial laboratory pellets ground to a powder and fed ad lib. (except in the pair-feeding studies described). Rats and mice were given commercial laboratory chow (Ralston Purina Co., St. Louis, Mo.); rabbits and chinchillas were given NIH Animal Feed A (Country Best Foods, Syracuse, N.Y.). Compounds to be added to the diet were dissolved in ether and mixed thoroughly with the ground chow; the ether was then evaporated on a steam bath.

Commercial phytol was obtained from several sources (Mann Research Laboratories, Inc., New York, N.Y.; Nutritional Biochemical Corp., Cleveland, Ohio; and K & K Laboratories, Inc., Plainview, N.Y.). According to GLC analysis, the purity of each lot was greater than 90%, but there were several minor contaminants in each. Oleyl (octadec-9-enyl) alcohol was obtained from Aldrich Chemical Co., Inc., Milwaukee, Wis., and oleic acid from Distillation Products Industries, Rochester, N.Y.

Phytanic acid was synthesized from phytol. A mixture of 75 g of commercial phytol, 0.2 g of platinum oxide, and 150 ml of ethanol was shaken overnight at room temperature under an atmosphere of hydrogen (40 psi). The reaction mixture was filtered and concentrated under reduced pressure to give material which was shown by GLC to be mostly dihydrophytol. This was dissolved in 200 ml of acetic acid, treated with 75 g of chromic acid in acetic acid, and left for 2 hr at 0°C and 1 hr at room temperature. Dilute hydrochloric acid and benzene were added to the mixture and the extract was evaporated under reduced pressure. The residual oil was partitioned between benzene and a solution of 5% sodium hydroxide in water–methanol 2:1. From the benzene layer, 30 g of neutral material was recovered. An oil that separated from the aqueous layer was acidified with hydrochloric acid and treated with benzene, which was evaporated to give 40 g of phytanic acid. The methyl ester of the latter was pure by GLC analysis. The product was distilled at 170°C/0.01 mm.

Extraction and Purification of Lipids

Serum lipids were extracted with chloroform–methanol 2:1 (25 ml/ml of serum). Two phases were formed by adding one-fifth volume of 0.02 N H_2SO_4, and an aliquot of the lipids from the chloroform phase was saponified for 1 hr at 60–80° C under nitrogen in 2% KOH in ethanol. Tissues were homogenized in ethanol–acetone 1:1 (25 ml/g), the homogenate was filtered, and the filtrate was taken to dryness. The residue was dissolved in alkaline ethanol and saponified as described above. Nonsaponifiable lipids were extracted from the alkaline saponification mixture into hexane (2 volumes, twice). Phytol-U-^{14}C, added to a lipid extract prior to saponification, was quantitatively recovered by this method.

Phytol was separated from other nonsaponifiable lipids by thin-layer chromatography (TLC) on Silica Gel G (Research Specialties Co., Richmond, Calif., or Allied Chemical Corp., New York, N.Y.), with benzene–ethyl acetate 4:1 was developer. Rhodamine 6G was incorporated in the silica gel slurry to allow location of phytol under UV light. Phytol was quantified by GLC of the free alcohol either on 10% ethylene glycol succinate polyester (EGS) or on 2% SE-30 (methyl polysiloxy gum, General Electric) on Gas-Chrom P, 80–100 mesh (Applied Science Laboratories Inc., State College, Pa.). Chromatographed on a 12 ft column containing EGS, at 180°C and 70 ml/min carrier gas flow rate, phytol had a retention time of 15.8 min.

After acidification of the saponified mixture and dilution to a final ethanol concentration of 30%, fatty acids were extracted with ether (equal volumes, three times). Methyl phytanate-^{14}C carried through the over-all procedure of saponification and extraction was quantitatively recovered in the saponifiable fraction. Fatty acids were methylated by heating at 60°C for 16 hr in 2–5% sulfuric acid in anhydrous methanol. When phytanic acid constituted a major component, it was readily quantified directly by GLC of the total mixture of fatty acid methyl esters on an EGS column under the same conditions as used for analysis of phytol. Methyl phytanate and methyl phytenate had retention times, relative to methyl stearate, of 0.83 and 1.47, respectively.

When present as minor components, phytanic acid and phytenic acid were purified prior to GLC by TLC on Silica Gel G or Adsorbosil-1 (Applied Science Laboratories Inc., State College, Pa.) slurried in 0.1% Rhodamine 6G. On development with benzene–hexane 2:1, methyl phytanate and methyl phytenate moved together just ahead of the band of saturated straight-chain fatty acid methyl esters, but the resolution was not complete. When palmitic acid-^{3}H and phytenic acid-U-^{14}C were carried through the methylation and TLC procedure, 90% of each was recovered in their respective TLC bands.

For resolution into lipid subclasses, an aliquot of the original chloroform extract was subjected to TLC in petroleum ether (or hexane)–diethyl ether–acetic acid 80:20:2. The individual zones were eluted with ether and with benzene–ethyl acetate 1:1, except for the phospholipid zone, which was eluted with methanol. An aliquot of each of the eluates was saponified and fatty acids were isolated and quantified as described above.

Histologic Preparations

Animals selected for histologic studies were taken from two control rats (220 g and 480 g), from two rats on 5% phytol for 12 and 19 days, respectively, and from one rat fed 2% phytol for 28 days and then 0.5% phytol for 236 days. They were deeply narcotized with chloroform. The chests were opened immediately thereafter and 0.4 ml of 10% heparin was injected into the right ventricle of the heart. The blood vessels were flushed with an aqueous salt solution and then perfused with Heidenhain's SUSA or Bouin's picric acid solution. The dissection was done 4–5 hr later. Pieces of tissue were placed in gelatin for preparation of frozen sections and Oil Red O staining, or in 95% ethyl alcohol for subsequent embedding in paraffin and periodic acid–Schiff–hematoxylin (PAS) staining. For determination of glycogen, sections were flattened on a slide covered with 80% ethyl alcohol. Two separate histologic procedures were used: (*a*) sections were left in 5% 5,5-dimethyl-1,3-cyclohexanedione in 80% alcohol for 3 hr and were subsequently stained with PAS (23); and (*b*) sections treated with PAS directly were compared with other sections previously digested with malt diastase at 42% for 1 hr, followed by PAS treatment.

RESULTS

Accumulation of Phytanic Acid and Phytenic Acid on Phytol-Containing Diets

As shown in Table 1, the feeding of diets containing 1–5% phytol by weight led to significant accumulation of phytanic acid in liver and serum of the four species tested. Analyses were in most cases done only on individual animals, and the reproducibility and range of the quantitative data have not been ascertained. The results show that the conversion of phytol to phytanic acid and accumulation of the latter, previously reported in the rat (1, 20), are not peculiar to that species. In the rat fed 5% phytol, phytanic acid was also present, but in lesser amounts in the intestine (9.9%), spleen (9.4%), kidney (4.6%), and brain (traces). No phytol was detected in the livers of the rats receiving 1% phytol, but it was present in the livers of two rats fed 5% phytol (0.5 and 4.5 mg/g) and in their intestines (0.10 and 0.12 mg/g). Since the animals were fed ad lib. to the time they were killed, this phytol could represent material recently absorbed. On the other hand, no dihydrophytol could be detected either in liver or intestine. In one rat, a trace of dihydrophytol was detected in the kidney (ca. 0.007 mg/g).

TABLE 1 ACCUMULATION OF PHYTANIC ACID IN LIVER AND SERUM OF FOUR MAMMALIAN SPECIES FED DIETS CONTAINING PHYTOL*

Species	Phytol in Diet	Days on Diet	Phytanic Acid as a Percentage of Total Fatty Acids	
			Liver	Serum
	%			
Rat, conventional	None	21	0†	0†
" "	1	21	2	2
" "	5	21	21	27
Rat, germ-free	5	12	12	—
" "	5	14	33	—
Mouse	5	7	13	6
Rabbit	5‡	25	64	40
Chinchilla	5	17	38	—
"	2	51	47	42

* All animals were on laboratory chow, to which phytol was added to make up indicated percentage by weight. Each value represents analytical results obtained on a single animal, except for the mouse, where tissues of two animals were pooled.

† Not detected with methods used; less than 0.2% of total fatty acids.

‡ 5% phytol diet for 25 days; then 2% phytol diet for additional 30 days.

The livers of rats on 5% phytol showed a definite yellow mottling. Total liver lipid concentration in one animal maintained for about 4 months on the diet was 95 mg/g versus 32 mg/g in a control. Increase in total lipid was accompanied by an increase in stainable fat in liver sections (see below).

To determine whether intestinal microorganisms might be responsible for the conversion of phytol to phytanic acid, we fed a pair of germ-free rats a 5% phytol diet. Because these rats were on a concentrated synthetic diet, the daily intake of phytol was somewhat smaller than that of the conventional rats fed the ground commercial chow. These two germ-free animals died after 12 and 14 days, respectively. Both animals showed very significant accumulation of phytanic acid in the liver, comparable to that in conventional rats fed 5% phytol (Table 1). Since one possible pathway from phytol to phytanic acid would involve phytenic acid as an intermediate, tissues of the germ-free rats were analyzed for phytenic acid. The livers contained highly significant amounts of phytenic acid (13% of total fatty acids in one animals and 17% in the other). The total carcass of one rat (minus the liver) was extracted and found to contain 239 mg of phytanic acid and 69 mg of phytenic acid. The lumbar plexes and the sciatic nerves were dissected from both rats and pooled for analysis (170 mg of nerve tissue). This nerve tissue contained phytanic acid (0.1 mg/g), but no detectable phytenic acid.

Phytanic acid was present in all the main lipid fractions of serum of a phytol-fed rat and was found at highest concentration in triglycerides and phospholipids (Table 2). On the other hand, phytenic acid was found in highest concentration in the cholesterol ester fraction,

TABLE 2 RELATIVE AMOUNTS OF PHYTANIC ACID AND PHYTENIC ACID IN THE FATTY ACIDS OF VARIOUS LIPID CLASSES IN THE SERUM OF A PHYTOL-FED RAT*

Lipid Fraction	Percentage of Total Fatty Acids in Fraction	
	Phytanic Acid	Phytenic Acid
Total fatty acids	15	3
Free fatty acids	10	0
Triglycerides	16	3
Mono- and diglycerides	6	0
Cholesterol esters	8	12
Phospholipids	15	1

* 5% phytol diet was started when the rat weighed 110 g, and was continued for 4 months. The growth rate of this animal was depressed but it continued to gain slowly despite the phytol intake and weighed 335 g at the time it was sacrificed.

TABLE 3 CHANGES IN CONCENTRATION OF LIVER AND SERUM PHYTANIC AND PHYTENIC ACIDS AFTER RETURNING A PHYTOL-FED RAT TO CONTROL DIET

Time on Diet	Phytanic Acid		Phytenic Acid	
	Liver	Serum	Liver	Serum
	mg/g	*μg/ml*	*mg/g*	*μg/ml*
21 Days on 5% phytol	5.1	211	0.9	37
21 Days on 5% phytol, then 12 days on control diet	0.9	30	0.6	1.5

and very little was present in the phospholipids; none was detected in free fatty acids or lower glycerides.

Mobilization of Stored Phytanic and Phytenic Acid

Two animals were fed the 5% phytol diet for 21 days; one was sacrificed at the end of this period and the other kept for an additional 12 days on the control diet before being sacrificed. The livers and sera of both rats were analyzed for phytanic and phytenic acid (Table 3). The absolute concentrations of phytanic acid in the liver and in the serum fell by over 80% during the 12 days after return to a control diet. These conventional rats, like the germ-free rats (Table 1), accumulated significant amounts of phytenic acid in liver and serum. The concentration of phytenic acid in the serum fell sharply on return to the control diet, but in the liver it decreased only slightly. In a second similar experiment two rats were fed the 5% phytol diet for 7 days, at the end of which one animal was sacrificed. Analysis of the liver showed that phytanic acid accounted for 4.9% of total fatty acids. The second animal was returned to the control diet for 9 additional days and was then killed. Its liver contained only 1.5% phytanic acid.

Growth Inhibition and Mortality

Growth of rats placed on a 5% phytol diet was severely impaired, with as much as a 50% mortality in the 1st month (Table 4). The food intake of the phytol-fed rats fell off soon after the experimental diet was started and averaged only about 50% that of the controls. In a second study, the control group was pair-fed with the experimental group. As shown in Table 4, the mean body weight in the two groups remained comparable, but four of the six phytol-fed rats died in 30 days, while there was no mortality in their pair-fed controls. Phytol at the 2% level inhibited but did not entirely prevent growth, and only one of six animals died in the 1st month. Another group of rats has been maintained on 0.5% phytol for over 15 months. At this dose there has been no inhibition of growth or evidence of toxicity.

Two rabbits on a 5% phytol diet lost weight rapidly. After a period of 25 days the serum fatty acid of one of the animals contained 30% phytanic acid. The phytol content of the diet was reduced to 2% at that time, but both rabbits died, after an additional 12 and 30 days, respectively.

Similar observations, i.e., rapid loss of weight and death occurring within 3 weeks, were made with two chinchillas on a 5% phytol diet.

The toxicity of phytol in mice is shown by the data in Fig. 1 and is discussed further below.

The close structural similarity between phytol and the side-chains of vitamin E and vitamin K suggested that phytol might interfere with the action of the fat-soluble vitamins. However, experiments with rats and mice showed that growth rate and mortality of animals fed 5% phytol diets were unaffected by dietary supplements of vitamins A, D, K, and E (A 30,000 U, D 4500 U, α-tocopherol 50–100 mg, K 20 mg—all per 100 g of diet).

TABLE 4 TOXICITY OF DIETARY PHYTOL IN RATS

Expt. No.	Phytol in Diet (by Weight)	Days on Diet	Mean Body Weight		Survivors	
			Controls	Phytol-Fed	Controls	Phytol-Fed
			g			
1	5% (Ad lib. feeding)	0	123	131	6	6
		10	172	130	6	6
		20	217	142	6	4
		30	254	161	6	3
		40	283	148	6	3
2	5% (Control rats pair-fed with phytol-fed group)	0	149	149	6	6
		10	135	139	6	5
		20	123	116	6	4
		30	154	160	6	2
3	2% (Ad lib. feeding)	0	60	58	6	6
		10	120	94	6	6
		20	184	140	6	6
		30	245	173	6	5

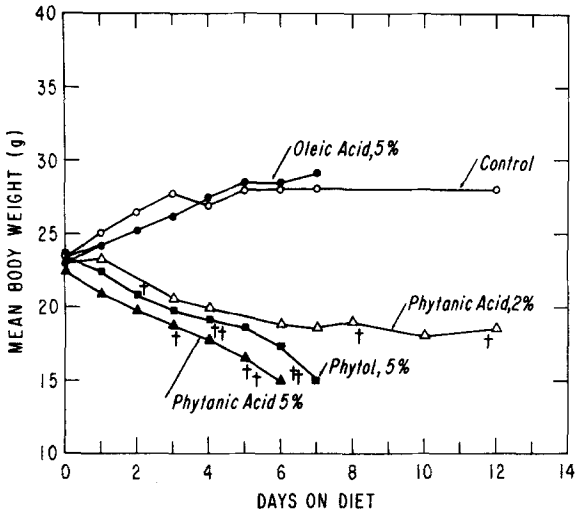

FIG. 1. Mean body weight as a function of time in groups of six mice each given the dietary additive indicated. The crosses each indicate the day of death of one animal of the group represented by the line above the cross.

Effects of Dietary Phytanic Acid

The toxic effects of phytol might be attributed to: (*a*) phytol per se, (*b*) metabolites of phytol other than phytanic acid, or (*c*) the phytanic acid formed from phytol. In the latter case, dietary phytanic acid, if adequately absorbed, might be expected to be as toxic as phytol, or more so. As shown in Fig. 1, 5% phytol and 5% phytanic acid fed to mice were roughly equivalent in the extent to which they impaired growth, and mortalities in the two groups were comparable. Two per cent phytanic acid appeared to be less toxic, but still impaired growth significantly. Survival time was prolonged, but three of six animals died within 2 weeks. Another group of mice placed on a 1.0% phytanic acid diet grew at a rate comparable to that in controls and there was no mortality in 6 weeks.

In order to compare the effects of feeding phytol and phytanic acid to those of a straight-chain alcohol and acid, we fed oleyl alcohol (5%) or oleic acid (5%) to mice. There was neither impairment of growth nor any signs of toxicity.

The concentration of phytanic acid in liver and serum of mice on phytanic acid diets is shown in Table 5. At the highest concentration used (5%), accumulation was rapid. By the 3rd day, phytanic acid accounted for 25% of the total liver fatty acids and 29% of the total serum fatty acids. An additional 4 days on the diet did not cause much further increase. No phytenic acid could be detected in these mice. The 2% phytanic acid diet fed for 14 days gave rise to similar liver and serum concentrations of phytanic acid. The concentrations were higher in the liver than in the serum. It is of interest that the 0.5% phytanic acid diet, although it led to appreciable concentrations of phytanic acid in liver and serum, did not impair growth.

TABLE 5 ACCUMULATION OF PHYTANIC ACID IN LIVER AND SERUM OF MICE ON DIETS CONTAINING PHYTANIC ACID*

Phytanic Acid in Diet (by Weight)	No. of Mice	Days on Diet	Phytanic Acid as Percentage of Total Fatty Acids	
			Liver	Serum
5%	2	3	24	28
	2	7	30	29
2%	1	11	35	8
	2	14	26	24
0.5%	2	17	17	8

* Results obtained from a single animal or from pooled samples of two or three animals in each case.

Changes in Nerve Function

In the course of the phytol-feeding experiments described above, the rats were periodically observed for appearance of neurologic signs. In no case were there overt signs of paralysis or muscular atrophy or any changes in gross motor coordination. The phytol-fed animals were able to balance themselves on a 1 cm straight edge and to climb a wire-mesh screen in a manner indistinguishable from that of the controls.

Electroretinograms were recorded at approximately weekly intervals in two or three rats of each group in the course of the paired-feeding study discussed above (Table 4, experiment 2). No abnormalities were detected in the course of the first 3 weeks on 5% phytol. Rats on 1% phytol were studied for almost 2 months and again no abnormalities were found. The eyes of one rat, sacrificed after 20 days on the 5% phytol diet, were examined histologically, and there were no signs of retinal degeneration.

Microscopic Examination

Liver. In the control animals, fat droplets were observed only in cells along the peripheral border of those lobules situated near the liver surface. In the deeper parts of the liver, no fat was demonstrable. In the animals treated with phytol, there was a moderate but distinct accumulation of fat in most cells throughout the entire liver, with more severe accumulation in a few cells. The Kupffer cells were rich in fat and often enlarged. All the fat granules observed stained uniformly red in frozen sections.

Since the perfusion technique with a cannula in the ascending aorta gives only spotty fixation of the liver, the intracellular content of glycogen can be reliably estimated only in those well-fixed parts in which the glycogen is evenly distributed in the liver cells. These areas were selected for comparison. In the control animals

most of the liver cells were profusely filled with glycogen. The content of glycogen was moderately decreased in the animal fed 0.5% phytol for 9 months while a severe depletion of glycogen was noted in the two animals kept on 5% phytol for a relatively short period.

Mitotic division in liver cells, absent in the control animals, was demonstrable in the two animals given 5% phytol; it occurred more frequently in the older of the two. None was seen in the animal fed a low concentration of phytol for 9 months.

Kidney. In the animal that received 0.5% phytol over a long period, scattered cells of the proximal tubules contained fat droplets, and the lumina of some distal tubules were filled with amorphic, faintly Oil Red O-stained masses.

Adrenals. In all the experimental animals, the cortical cells were rich in fat.

Brain and Spinal Cord. In the animal receiving 0.5% phytol for a long period, scattered cells in the leptomeninges contained slightly more fat granules than in the control animals. In one rat treated with 5% phytol for a short period, scattered endothelial cells lining the perineural capsule contained considerable amounts of fat. Otherwise, no characteristic changes were demonstrable in cross sections of the peripheral nerves of any of the animals. The neurons of the cerebral cortex and the ependymal cells covering the ventricles did not show any accumulation of fat.

DISCUSSION

These results establish in four species that dietary phytol is converted to phytanic acid and stored when sufficiently high concentrations are fed. In the rat, unchanged phytol also accumulates. Feeding of phytanic acid in mice leads to accumulation of phytanic acid in tissues. Hansen, Shorland, and Prior (16) and Klenk and Kremer (21) have independently made similar observations on the effect of feeding phytanic acid in the rat. The latter have also shown that feeding dihydrophytol causes accumulation of phytanic acid. Billeter, Bolliger, and Martius (24) have shown in their study on the metabolism of vitamin K that the isoprenoid side chain of phylloquinone is split by intestinal bacteria of animals and that the metabolite recovered from breast muscle of the pigeon is probably phytanic acid. Lough (7) has suggested that the relatively large amounts of phytanic acid in bovine serum may be derived from dietary chlorophyll.

Studies in this laboratory show that in the rat (1, 20, 22) and in normal man (17), tracer doses of phytol-U-^{14}C are very readily oxidized and in part converted to phytanic acid. More recently it has been shown that phytanic acid is at least as rapidly oxidized as phytol (22). Accumulation evidently depends, then, upon the intake's exceeding the capacity of the animal to metabolize phytol and (or) phytanic acid. In the present studies phytanic acid from the tissues of phytol-fed rats disappeared rather rapidly when phytol was removed from the animal's diet. While these two experiments may not justify quantitative conclusions, they suggest that the normal rat can quite rapidly metabolize large stores of phytanic acid. This capacity has also been indicated by the studies of Hansen et al. (16), and by Klenk and Kremer (21). In contrast, patients with Refsum's disease, while they readily convert labeled phytol to phytanic acid (17, 19), oxidize the substrate poorly and show high levels of labeled phytanic acid in plasma long after it has disappeared from the plasma of normal subjects similarly studied (17). Presumably the metabolic error lies in a relative inability to break down the branched-chain structure of phytanic acid (17).

The finding of phytenic acid in significant amounts in this study, and also by Klenk and Kremer (21) in the tissues of phytol-fed rats, shows that phytol can be oxidized to the carboxylic acid prior to reduction of the Δ^2-double bond. Subsequent hydrogenation would then yield phytanic acid, a conversion demonstrated in this laboratory (22). These results do not, of course, rule out the alternative sequence of reactions: reduction to dihydrophytol prior to oxidation of the alcohol function to the carboxyl function. The isolation of small amounts of phytenic acid from the urine of a rabbit to which phytol was intramuscularly administered was indicated in an earlier study by Fischer and Bielig (25), although these authors could not positively identify the product. That the conversion of phytol to phytanic acid does not depend absolutely upon the action of intestinal microorganisms is shown by the fact that the accumulation of phytanic acid in germ-free rats was comparable in magnitude to that in conventional rats. The absence of any detectable phytenic acid in serum of rats fed phytanic acid suggests that desaturation may not occur, or occurs to only a limited extent.

In animals fed phytol, a moderate accumulation of Oil Red O-stainable fat in liver cells was the only significant change microscopically demonstrable. Even more severe fatty liver was noted at autopsy in the original patients afflicted with Refsum's disease (26–28), and in two more recent cases in which an increased concentration of liver phytanic acid has been demonstrated (4, 29). The increased fat content of the liver was originally interpreted to be an expression of an abnormal lipid metabolism (28). The lipid accumulation in the phytol-fed rat may be analogous, in this case induced by an extremely high intake of a lipid for which the animal has an appreciable but limited degradative capacity. However, fat accumulation in the liver occurs under many diverse experi-

mental conditions and may merely reflect, in part, the poor nutritional state of the animals. In any case, the degree of fatty liver was almost certainly not severe enough to account for the observed toxic effects.

The significance of two other observations in the liver, namely karyokinesis and glycogen depletion, may be nonspecific. The former may be associated with hypertrophy of the liver and the latter may be due to the reduced food intake when animals are on phytol-containing diets.

The cause of growth impairment and death on high intakes of phytol or of phytanic acid observed in this study, as well as in others (16, 21), is not known. Diets containing 5% oleyl alcohol or 5% oleic acid did not impair growth of mice.

In view of the isoprenoid structure of phytanic acid, one might speculate that it could competitively interfere with the synthesis or function of ubiquinones, vitamin K, or vitamin E. The failure, in the present studies, of supplements of the fat-soluble vitamins to counteract the toxic effects does not entirely rule out this possibility, since competition at the site of action might still be highly effective. Interference with sterol biosynthesis, which depends on isoprenoid intermediates, is not involved, since it has been shown that cholesterol biosynthesis from mevalonate occurred at a normal rate in a patient with Refsum's disease who had a relatively high plasma phytanate concentration (17).

In none of the animals studied at any dose of phytol or phytanic acid were there any grossly detectable manifestations of neurological damage. There were no functional abnormalities revealed by the electroretinograms in rats, nor were there any signs of retinal degeneration detectable histologically. Moreover, none of the animals on a phytol diet manifested any structural changes in the peripheral and central nervous system that are characteristic of Refsum's disease (26–28). In short, despite the marked accumulation of phytanic acid in peripheral tissues, it was not possible under the conditions used to mimic the changes seen in patients with Refsum's disease. It should be noted that ruminants normally have phytanic acid in their plasma, up to 5% of total fatty acids (7–9), and do not manifest gross neurologic damage or other signs of toxicity.

The negative evidence presented here does not rule out a causative relationship between tissue accumulation of phytanic acid and the functional and structural abnormalities in Refsum's disease. In the clinical disease, symptoms develop slowly and progressively over many years. The dosages of dietary phytol or phytanic acid needed to produce tissue accumulation of phytanic acid in normal animals are large and incompatible with growth and survival in the species tested. Moreover, even on the highest dosage of dietary phytol the concentration of phytanic acid reached in the peripheral nerves was only 0.1 mg/g while that reported from postmortem analysis of the sciatic nerve of a patient with Refsum's disease was about 8 mg/g (29). On the other hand, the dosages of dietary phytol that *can* be tolerated, and thus permit long-term studies, cause only a very limited build-up of tissue phytanic acid; the negative experiments in the rats maintained on low dosages for a long period of time may therefore not represent a critical test. Furthermore, there may be an important species difference in the vulnerability of the nervous system to changes induced by phytanic acid or its metabolites. Until it is possible to maintain higher levels of phytanic acid over a long period of time, no final conclusion should be drawn concerning the possible cause and effect relationship between this unusual fatty acid and the pathologic changes observed in Refsum's disease.

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